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Phylogeny and biogeography of *Crinum* L. (Amaryllidaceae) inferred from nuclear and limited plastid non-coding DNA sequences

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The genus *Crinum* L. is the only pantropical genus of the Amaryllidaceae. Phylogenetic and biogeographical analyses of nrDNA ITS and plastid *trnL-F* sequences for all continental groups of the genus *Crinum* and related African genera are presented, with the genus *Amaryllis* used as outgroup. ITS indicates that *C. baumii* is more closely related to *Ammocharis* and *Cybistetes* than to *Crinum sensu stricto*. Three clades are resolved in *Crinum s.s.* One unites a monophyletic American group with tropical and North African species. The second includes all southern African species and the Australian endemic *C. flaccidum*. The third includes monophyletic Madagascar, Australasian and Sino-Himalayan clades, with southern African species. The *trnL-F* phylogeny resolves an American and an Asian/Madagscar clade, and confirms the relationship of *C. flaccidum* with species endemic to southern Africa. The salverform, actinomorphic perianths of subg. *Crinum* appear to have evolved several times in the genus from ancestors with zygomorphic perianths (subg. *Codonocrinum*), thus neither subgenus is monophyletic. Biogeographical analyses place the origin of *Crinum* in southern Africa, as the region is optimized at all ancestral nodes in the tree topology, and in basal interior nodes of all but one of the major clades. The genus underwent three major waves of radiation corresponding to the three main clades resolved in our trees. Two entries into Australia for the genus are indicated, as are separate Sino-Himalayan and Australasian dispersal events. *Botanical Journal of the Linnean Society*, 2003, **141**, 349–363.

ADDITIONAL KEYWORDS: molecular systematics – Africa – geophytes – dispersal – cladistics – monocotyledons.

INTRODUCTION

The genus *Crinum* L. is the only pantropical genus of the Amaryllidaceae, with species occurring in Africa, America, Asia, and Australia. *Crinum* has seeds well adapted for oceanic dispersal (Koshimizu, 1930), which are considered to have engendered its broad distribution (Arroyo & Cutler, 1984). The genus is most speciose in Africa, particularly sub-Saharan Africa (Nordal, 1977), and its systematic affinities are with a group of entirely African endemic genera, constituting the tribe Amaryllideae (Snijman & Linder, 1996; Meerow & Snijman, 1998, 2001). This tribe is extremely well-marked by numerous morphological synapomorphies, such as extensible fibres in the leaf tissue, bisulculate pollen with spinulose exines, scapes with a sclerenchymatous sheath, unitegmic or ategmic ovules, and nondormant, water-rich, nonphytomelanous seeds with chlorophyllous embryos (Snijman & Linder, 1996). In plastid DNA sequence analyses of the Amaryllidaceae (Ito *et al*., 1999; Meerow *et al*., 1999), this tribe is the first branch to resolve in the family and receives high bootstrap support (>90%).

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Crinum was established by Linneaus (1753). Herbert (1837) divided the genus into two sections based on the degree to which the tepals are patent. Baker (1881) divided the genus into three subgenera: *Stenaster*, with salverform, actinomorphic perianths, straight floral tubes and linear segments; *Platyaster*, similar to the former but with lanceolate segments; and *Codonocrinum* with funnel-form, zygomorphic flowers and curved tubes. He later (Baker, 1898) submerged *Platyaster* into subgenus *Stenaster*, which must be called subgenus *Crinum* since it contains the type species, *C. americanum* L.

Crinum was not thoroughly monographed in the last century, except for a synoptic update of Baker (1881) by Uphof (1942). It has been the subject of regional treatments (e.g. Hepper, 1968; Geerinck, 1973; Verdoorn, 1973; Nordal, 1977, 1982, 1986; Nordal & Wahlstrom, 1980; Lehmiller, 1997a,b) and it is estimated that the genus contains 60–70 species (Fangan & Nordal, 1993). Fangan & Nordal (1993) used DNA RFLPs to investigate the phylogeny of seven species. Meerow & Snijman (2001) included four species in their ITS analysis of the tribe Amaryllideae. In this paper, we investigate the phylogeny of 38 species of *Crinum*, representing all of the continental groups within the genus, using nuclear rDNA ITS sequences (ITS1 spacer, 5.8S intron, ITS2 spacer), and plastid *trnL-F* sequences, and discuss the results in a biogeographical and evolutionary context.

MATERIAL AND METHODS

Sampling

Genomic DNA was extracted from silica gel-dried leaf tissue of the taxa listed in Table 1, as described by Meerow *et al*. (2000).

DNA extraction, amplification and sequencing protocols

The *trnL-trnF* region was amplified and sequenced using the primers of Taberlet *et al*. (1991) as described by Meerow *et al*. (1999). Amplification of the ribosomal DNA ITS1/5.8S/ITS2 region was accomplished using flanking primers (18S, 26S) AB101 and AB102 (Douzery *et al*., 1999), and the original White *et al*. (1990) internal primers ITS2 and 3 to amplify the spacers along with the intervening 5.8S intron as described by Meerow *et al*. (2000). All polymerase chain reaction (PCR) amplifications were performed on an ABI 9700 (Perkin-Elmer Applied Biosystems, Foster City, California, USA).

Amplified products were purified using QIAquick (Qiagen, Valencia, California, USA) columns, following the manufacturer's protocols. Cycle sequencing reactions were performed directly on purified PCR products on the ABI 9700, using standard dideoxy-cycle protocols for sequencing with dye terminators on either an ABI 377 or ABI 310 automated sequencer (according to the manufacturer's protocols; Applied Biosystems, Foster City, California, USA).

Sequence alignment

Both the ITS and *trnL-F* matrices contained few gaps and were readily aligned manually using Sequencher 4.1 (Gene Codes, Ann Arbor, Michigan, USA). The alignment is accessible through GenBank or from the first author.

Phylogenetic analyses

The ITS matrix consisted of 43 taxa (38 *Crinum* species, two species of *Amaryllis* and *Ammocharis*, and *Cybistetes longifolia*). *Amaryllis* was designated as outgroup. *Amaryllis* is sister to all other members of the tribe Amaryllideae (Meerow & Snijman, 2001). *Ammocharis* and *Cybistetes* are the only other members of the subtribe Crininae that together form a sister clade to *Crinum* (Meerow & Snijman, 2001). The plastid *trnL-F* matrix consisted of 19 taxa, 16 species of *Crinum* and one species each of *Amaryllis* (*A. belladonna*), *Ammocharis* (*A. coranica*), and *Cybistetes longifolia*. We only sampled one to several taxa from each of the clades resolved by ITS, and saw little likelihood that additional sampling would increase information content of the *trnL-F* data matrix. In the combined data set, taxa with only one sequence were coded as missing data for the absent sequence. Aligned matrices were analysed using the parsimony algorithm of PAUP* for Macintosh (version 4.0b10; Swofford, 1998), with the MULPARS option invoked. Tree branches were retained only if unambiguous support was available (i.e. branches were collapsed if the minimum length = 0). The few gaps were coded as missing characters, as developing a binary-coded strict homology gap matrix added no further resolution to the trees in preliminary analyses.

For all matrices, a heuristic search was conducted under the Fitch (equal) weights (Fitch, 1971) criterion with 1000 random sequence additions (Maddison, 1991) and tree bisection and reconnection (TRB) branch swapping. We permitted only 20 trees to be held at each step to reduce the time spent searching trees at suboptimal levels. All minimal trees collected in the 1000 replicates were then swapped to completion.

We combined the data matrices, opting for the total evidence approach (Seelanan, Schnabel & Wendel, 1997; Dubuisson, Hebant-Mauri & Galtier, 1998). Though the *trnL-F* matrix yielded only a few parsi-

mony informative base substitutions, it nonetheless confirmed some of the relationships well-supported by the ITS matrix, and inclusion in this study is therefore warranted. However, before combining the ITS and *trnL-F* data sets, we performed a partition homogeneity test on the matrices (Farris *et al*., 1994, 1995) to assess the degree of congruence between them. Five hundred heuristic searches were conducted, each with ten random addition replications, saving 20 trees from each for TBR branch-swapping.

Internal support was determined by bootstrapping (Felsenstein, 1985; 5000 replicates with simple addition) and calculation of Bremer (1988) decay indices (DI) using the program TreeRot vs. 2.1 (Sorenson, 1996). The cut-off bootstrap percentage is 50. A bootstrap value greater than 75% was considered good support, 65–75% was designated moderate support, and less than 65% as weak (Meerow & Snijman, 2001; Meerow *et al*., 2002). Five hundred replicate heuristic searches were implemented for each constraint statement postulated by TreeRot, saving ten trees per replicate. A minimum DI = 2 was considered to represent good support for a clade.

Biogeographical analyses

The biogeographical patterns inferred from our gene trees were assessed using both Fitch optimization (Maddison, Ruvolo & Swofford, 1992) with MacClade version 4.03 (Maddison & Maddison, 2001) and the dispersal-variance method of analysis (Ronquist, 1997) using the program DIVA version 1.1 (Ronquist, 1996). The program uses vicariance (i.e. allopatric speciation) in its optimization of ancestral distributions but takes into consideration dispersal and extinction events, and indicates their direction (Ronquist, 1996, 1997). The most-parsimonious reconstructions minimize such events. Unlike Fitch optimization, DIVA does not restrict widespread distributions to terminals or limit ancestral distributions to single areas (Ronquist, 1996). DIVA requires a fully bifurcated tree for analysis, thus one tree (with zero-length branches not collapsed) from the combined sequence analysis was used for optimization of 15 coded geographical areas (Table 1). It must be noted that this tree was one of 150 equally parsimonious topologies, and contains zero-length internal branches. Fitch optimization of area data was performed on the same tree using a single multistate character (Table 1). Two DIVA analyses were performed, one without limits on the maximum areas allowed for ancestral nodes (15), and another in which the limit was set to the minimum (2) to reduce ambiguities at the more basal nodes of the tree. An exact optimization (vs. heuristic) was invoked in both analyses by allowing the maximum number of alternative reconstructions to be held at any node.

RESULTS

ITS

Of the 807 characters (ITS1, 5.8S intron, ITS2) included in the analyses, 122 were parsimony informative. The search found 119 equally most-parsimonious trees of length = 322, consistency index (CI) = 0.79 and retention index (RI) = 0.89.

Two very well-supported clades are resolved by ITS (Fig. 1). The smaller of the two, with a bootstrap of 100% and DI = 11 unites *Crinum baumii* as sister to *Ammocharis* and *Cybistetes*. The position of *Cybistetes* as sister to *Ammocharis* is only weakly supported (bootstrap = 58%, DI = 1).

The second, larger clade with strong support (bootstrap = 99, DI = 9) consists of all of the remaining *Crinum* species. Three subclades of *Crinum* species form a trichotomy within this larger group. The first (A), with a bootstrap = 64 and DI = 2, includes a well-supported American group (bootstrap = 99%, DI = 7) embedded among strictly tropical and north African species. No species endemic to South Africa included in the analysis resolves within this clade. *Crinum distichum*, *C. humile* and *C. jagus* form a well-supported group (bootstrap = 100%, DI = 9), as does *C. kirkii* and *C. politfolium* (bootstrap = 90%, DI = 3).

The second subclade (B) is the best supported of the three (bootstrap = 91%, DI = 4). With the exception of the Australian endemic *C. flaccidum*, the clade consists entirely of African species, including the only South African endemics (e.g. *C. acaule*, *C. bulbispermum*, *C. campanulatum*, *C. moorei*, *C. variabile*) included in the analysis. Outside of well-supported sister relationships between *C. acaule* and *C. forbesii*, and between *C. bulbispermum* and *C. variabile*, the clade is largely unresolved.

The third subclade (C), with a bootstrap = 63 and DI = 2, unites the Asiatic species with the Madagascar endemics. The West African *C. fimbriatulum* is sister to all other members of the clade. The Sino-Himalayan species (*C. defixum* and *C*. sp., China) and the Australasian species form separate subclades, both with a bootstrap of 65% and DI = 1. Three of the four Australasian species form a well-supported monophyletic group with a bootstrap of 99% and DI = 5. The Madagascar species resolve as monophyletic with moderate support (bootstrap = 73%, DI = 2). They form a polytomy with the Australasian group, and two broadly distributed African species, *C. subcernuum* (Mozambique, Namibia, Tanzania) and *C. buphanoides* (southwest Africa, Angola, Transvaal region of South Africa).

Plastid *trnL-F*

Of the 940 characters included in the analysis, only eight were parsimony informative. Five equally

Table 1. Species, voucher specimens, GenBank sequence accession numbers (or previous citation), and geographical area codes used in the phylogenetic analyses of *Crinum*. Vouchers are deposited at FTG unless otherwise stated

Taxon	Voucher specimen	Provenance	GenBank accession no. or literature citation		Area code[1]
			ITS	*trnL-F*	
Amaryllis belladonna L.	M. W. Chase 612 (K)	Western Cape, South Africa	Meerow & Snijman (2001)		A
A. paradisicola Snijman	van Jaarsveld 13263 (NBI)	Western Cape, South Africa	Meerow & Snijman (2001)		A
Ammocharis coranica Herb.	Meerow 2320	Eastern Cape, South Africa	Meerow & Snijman (2001)	AY139152	ACE
A. nerinoides (Baker) Lehmiller	Meerow 2321	Namibia	AY139116		B
Crinum abyssinicum Hochst. ex A. Rich.	Meerow 2322	Ethiopia	AY139117	AY139153	D
C. acaule Baker	Meerow 2338	Natal, South Africa	AY139118		A
C. americanum L.	Meerow 2323	Florida, USA	AY139119	AY139154	G
C. asiaticum L.	Meerow 2334	cultivation, Florida, USA	AY139120	AY139155	M
C. baumii Harms.	Van Zyl 99.B (PRE)	Namibia	AY139121	AY139156	B
C. broussonetii (Redouté) Herb.	Meerow 2324	Chad	AY139122		C
C. bulbispermum (Burm.) Milne-Redhead & Schweickerdt	Meerow 2339	Natal, South Africa	AY139123		A
C. buphanoides Welw. ex Baker	Meerow 2325	Transvaal, South Africa	AY139124	AY139157	AB
C. campanulatum Herb.	Meerow 2337	Eastern Cape, South Africa	Meerow & Snijman (2001)	AY139158	A
C. carolo-schmidtii Dinter	Meerow 2340	Namibia	AY139125		A
C. crassicaule Baker	Meerow 2341	Namibia	AY139126		A
C. cruentum Ker Gawl.	T. M. Howard s. n.	Oaxaca, Mexico	AY139127	AY139159	G
C. defixum Ker Gawl.	Traub 1235 (MO)	Nepal	AY139128		L
C. distichum Herb.	Meerow 2326	Chad	AY139129	AY139160	C
C. erubescens Sol.	T. M. Howard s. n.	Brazil	AY139130	AY139161	HI
C. fimbriatulum Baker	Leach 14510 (PRE)	Angola	AY139131		B
C. flaccidum Herb.	Meerow 2327	Australia	AY139132	AY139162	N
C. forbesii (Lindl.) Schult. emend. Herb.	Meerow 2328	Transvaal, South Africa	AY139133	AY139163	A

C. humilis A. Chev.	Meerow 2329	Cameroun	AY139134		C
C. jagus Thomps.	Meerow 2330	cultivation, Florida, USA	AY139135	AY139164	C
C. kirkii Baker	Meerow 2342	Tanzania	AY139136		C
C. latifolium Andr.	Meerow 2343	India	AY139137		O
C. ligulatum Baker	Hardy 2995 (PRE)	Madagascar	AY139138	AY139165	F
C. macowanii Baker	Meerow 2344	Kenya	Meerow & Snijman (2001)		AE
C. mauritianum Lodd.	Hardy s. n. (PRE)	Madagascar	AY139139		F
C. modestum Baker	Meerow 2345	Madagascar	AY139140		F
C. moorei Hook. F.	Meerow 2346	Natal, South Africa	AY139141		A
C. oliganthum Urb.	Meerow 2336	Cuba	AY139142	AY139166	J
C. pedunculatum R. Br.	Meerow 2335	Australia	AY139143	AY139167	N
C. politifolium R.Wahlstr.	Meerow 2347	Tanzania	AY139144		C
C. razafindratsiri Lehmiller	Lehmiller 1944 (TAMU)	Madagascar	AY139145		F
C. subcernuum Baker	Meerow 2348	Namibia	AY139150		BE
C. variabile Herb.	Meerow 2331	Western Cape, South Africa	Meerow & Snijman (2001)		A
C. venosum Baker	Meerow 2349	Northern Australia	AY139146		N
C. yemense Deflers	M. W. Chase 1595 (K)	Yemen	AY139151	Meerow *et al.* (1999)	D
C. sp., Borneo	Meerow 2332	Borneo	AY139147	AY139168	M
C. sp., SW China	Meerow 2333	Yunnan, China	AY139148		K
C. sp., Peru	Schunke 14054	Peru	AY139149		I
Cybistetes longifolia (L.) Milne-Redh. & Schweick.	Duncan 304 (NBG)	Western Cape, South Africa	Meerow & Snijman (2001)	AY139169	A

[1]A = South Africa, B = South-west Africa, C = tropical Africa, D = North Africa, E = East Africa, F = Madagascar, G = North America, H = Central America, I = South America, J = Cuba, K = China, L = Nepal, M = Australasia (south-east Asia and Pacific), N = Australia, O = India.

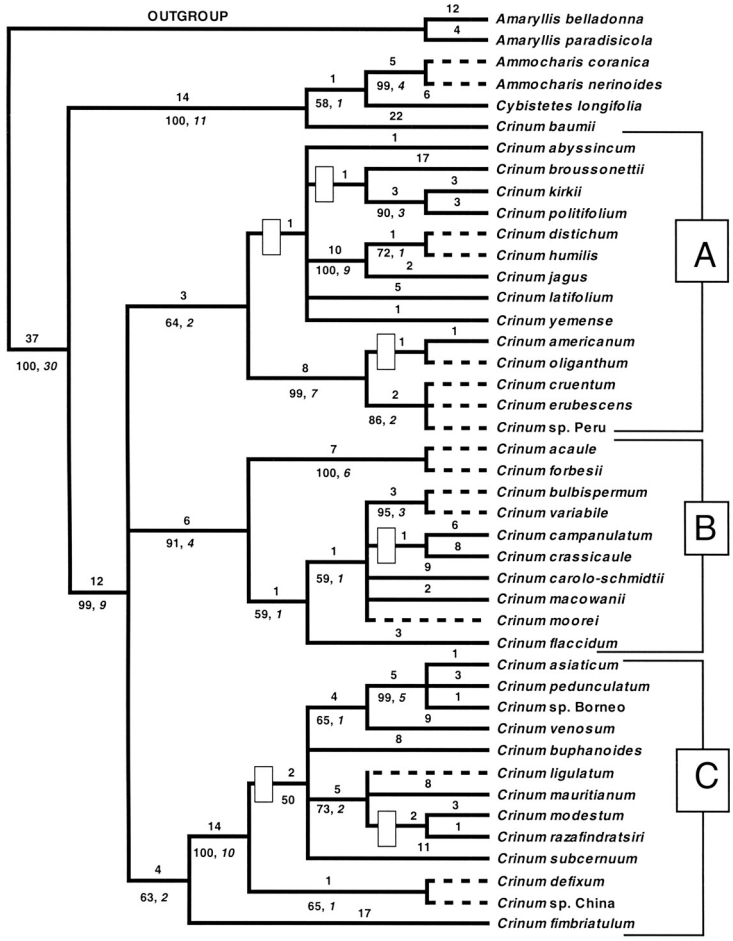

Figure 1. One of 119 most-parsimonious trees found by phylogenetic analysis of nrDNA ITS sequences across 43 species of *Crinum* and related genera. Numbers above branches are branch lengths. Numbers below branches are bootstrap percentages and decay indices (italic), respectively. Dashed lines are zero-length branches. A white bar across a branch signifies a collapsed node in the strict consensus of all trees.

parsimonious trees were found of length = 42 steps, CI = 1.00, RI = 1.00 (Fig. 2). The tree is mostly unresolved, but four clades are supported. A tropical Australasian clade (bootstrap = 64%, DI = 1) is resolved as sister to the single Malagasy species included, *C. ligulatum* (bootstrap = 64%, DI = 1). An American clade is resolved with a bootstrap of 62% and DI = 1. Finally, South African *C. campanulatum* and Australian *C. flaccidum* form a monophyletic group with a bootstrap of 92% and DI = 2.

Combined analysis

The *P*-value = 0.995, indicating a high level of congruence between the ITS and *trnL-F* matrices, though this value is compromised by the lower sampling level for *trnL-F* compared to ITS, and relative paucity of informative base substitutions in the plastid data. A total of 150 equally parsimonious trees were found by the heuristic search of length = 363, CI = 0.81, RI = 0.89. Not surprisingly, the trees (Fig. 3) are very similar in topology to those found using ITS alone. However, bootstrap support for the position of *C. flaccidum* as sister to an entirely African clade rises to 87%, whereas support for the Sino-Himalayan clade drops to 58%. The position of this clade as sister to the Australasian–Madagascar–*C. buphanoides*–*C. subcernuum* clade is slightly better supported and does not collapse in strict consensus.

Biogeographical analysis

While allowing the maximum area assignments (15) to ancestral nodes in DIVA produced an area optimization requiring only 17 dispersal events, it did so by assigning the more basal nodes every possible area.

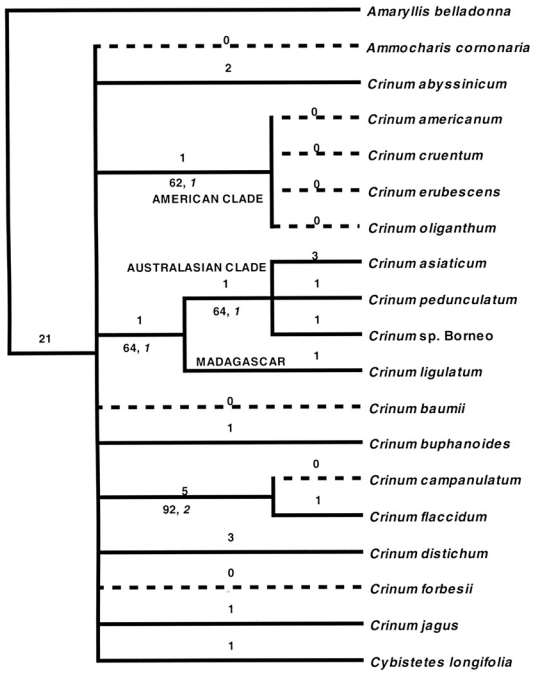

Figure 2. One of five most-parsimonious trees found by phylogenetic analysis of plastid *trnL-F* sequences across 19 species of *Crinum* and related genera. Numbers above branches are branch lengths. Numbers below branches are bootstrap percentages and decay indices (italic), respectively. Dashed lines are zero-length branches.

Constraining the program to maxareas = 2 yields a more realistic scenario, but the cost raises the number of dispersal events to 23. The latter optimization is referenced in the discussion (Fig. 4). Even with maxareas set to 2, one node was assigned four equally parsimonious areas, and one received eight possible assignments.

Both Fitch optimization and DIVA were largely congruent (Fig. 4). However, Fitch optimization places the origins of the lineage in South Africa, while DIVA found an origin in south-western Africa equally parsimonious. The ancestral origin of clade A is equivocal by Fitch optimization, and DIVA assigns eight possible area assignments, all of which include either South Africa or south-western Africa and respective dispersal to tropical Africa (C) North Africa (D), North America (G), and (J) Cuba. However, clade A is the only one of the three from which South Africa disappears from either the internal nodes or a terminal distribution (the distribution of *C. buphanoides* in clade C includes South Africa). In clade B, South Africa persists as an area component through internal nodes as well (Fig. 4). Clade B contains most of the endemic South African species included in the analyses. Clade C is rooted in south-western Africa by *C. fimbriatulum* (subg. *Codonocrinum*, Figs 1,3,4), but at the next node, a Sino-Himalayan clade with actinomorphic subg. *Crinum* flower morphology (Fig. 5) is sister to an entirely subg. *Crinum* clade, including two species from southern Africa (*C. buphanoides* and *C. subcernuum*) whose exact relationships are unresolved. (Figs 1,3).

DISCUSSION

Meerow *et al*. (1999), using three plastid DNA sequences, found that the Amaryllideae, the tribe of Amaryllidaceae to which *Crinum* belongs, was the most robustly supported clade in their parsimony topologies and was sister to the rest of the Amaryllidaceae. Meerow & Snijman (2001), combining morphological characters and ITS sequences, analysed the Amaryllideae and resolved a well-supported monophyletic subtribe, Crininae, consisting of *Crinum*, *Ammocharis* and *Cybistetes*. Only four *Crinum* species were included in that analysis. *Crinum* is the largest genus in the tribe, and the only one to disperse outside of Africa. The fleshy, floating, and salt-resistant seed of *Crinum* has been implicated as the likely agent of its dispersal success (Koshimizu, 1930; Arroyo & Cutler, 1984).

Fangan & Nordal (1993) performed restriction fragment length polymorphism (RFLP) analysis on seven species of *Crinum*, using *Pancratium canariensis* as outgroup. *Pancratium*, as the authors concede, was probably not the best choice of outgroup for the polarization of character states in *Crinum*, as it is only distantly related to the ingroup. In fact, it is part of the monophyletic Eurasian clade of Amaryllidaceae that branches more terminally than Amaryllideae in plastid DNA based trees of the entire family (Meerow *et al*., 1999). Despite the limited sampling, Fangan & Nordal's (1993) RFLP topology resolved some geographically congruent clades. A West African species clade (*C. glaucum*, *C. jagus* and *C. zeylanicum sensu* Nordal) resolved as a monophyletic sister group to a clade inclusive of the East African and Indo-Pacific species that they sampled (2 spp.). *Crinum asiaticum* (subg. *Crinum*) resolved as sister to *C. latifolium* (subg. *Codonocrinum*) within this latter clade. These results were in contrast to their accompanying cladistic analysis of the same species across 11 morphological characters with the genus *Ammocharis* used as outgroup. In these analyses, the species of subgenus *Codonocrinum* formed a monophyletic group that was sister to *C. asiaticum*, the only representative of subg. *Crinum* included. Moreover, morphology placed *C. zeylanicum* and *C. latifolium* in the same trichotomous clade with *C. politifolium*. The former two species have had a long and confused taxonomic history (Nordal, 1977; Dassanayake, 1981) in terms of the

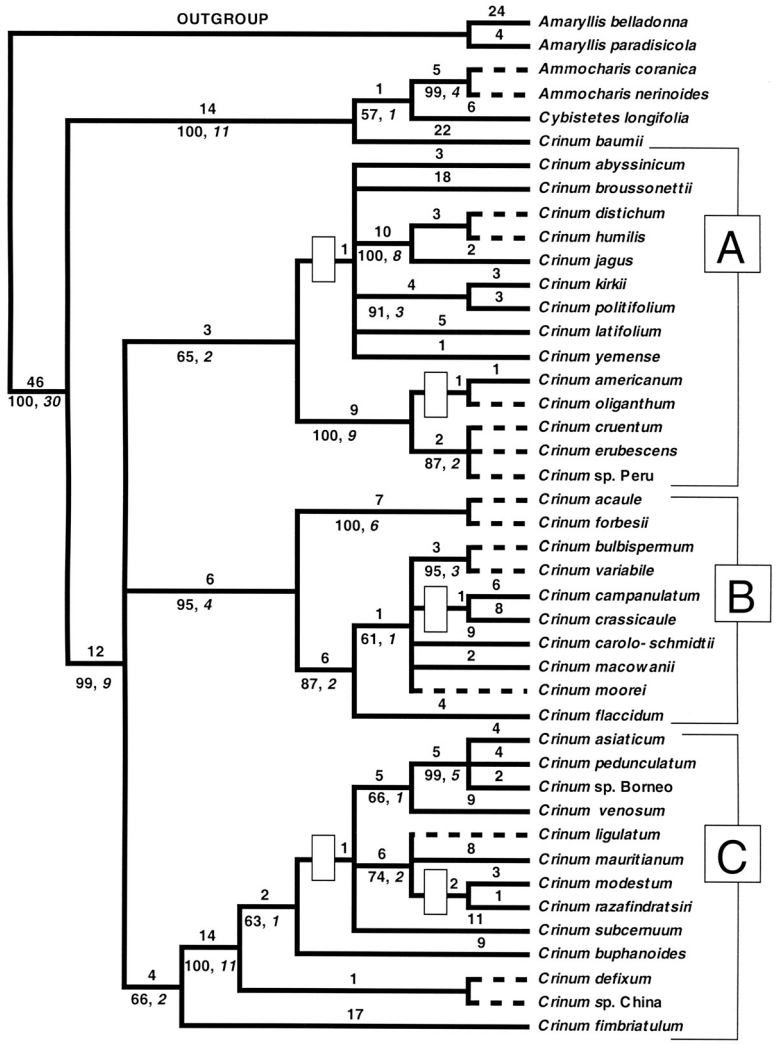

Figure 3. One of 150 most-parsimonious trees found by phylogenetic analysis of combined nrDNA ITS and plastid *trnL-F* sequences across 43 species of *Crinum* and related genera. Numbers above branches are branch lengths. Numbers below branches are bootstrap percentages and decay indices (italic), respectively. Dashed lines are zero-length branches. A white bar across a branch signifies a collapsed node in the strict consensus of all trees.

identity of *C. latifolium* relative to *C. zeylanicum* (Fangan & Nordal, 1993). *Crinum latifolium* as treated by Fangan & Nordal (1993) is the only *Codonocrinum* species found outside Africa other than *C. flaccidum* that has not yet been proven to have been introduced by humans. To confuse matters further, the Linnean name *C. latifolium* is sometimes erroneously applied to a *Crinum* in southern China with radially symmetrical, salverform flowers (Zhanhe & Meerow, 2001). In our analyses, *C. latifolium*, despite its occurrence in India and Sri Lanka, is well nested in the tropical African/American clade (clade A, Figs 1,3,4). There are many tropical East African *Crinum* species that are similar in morphology to *C. latifolium*, and a putative scenario of how this species arrived in India and Sri Lanka is discussed later in this paper. Finally, Fangan & Nordal advised that, on the basis of their RFLP topology, the characteristic floral morphology of either subgenera *Crinum* or *Codonocrinum* may have evolved more than once or reversals for this character were possible.

Our ITS (Fig. 1) and combined (Fig. 3) phylogenies support Fangan & Nordal's (1993) suggestion based on RFLPs that neither morphologically based subgenus *Crinum* nor *Codonocrinum* is monophyletic. At issue is which state is plesiomorphic in the genus. Snijman & Linder (1996) concluded that zygomorphy is the ancestral state in tribe Amaryllideae, and Meerow *et al*. (1999) hypothesized that perianth symmetry in Amaryllidaceae is under simple genetic control and

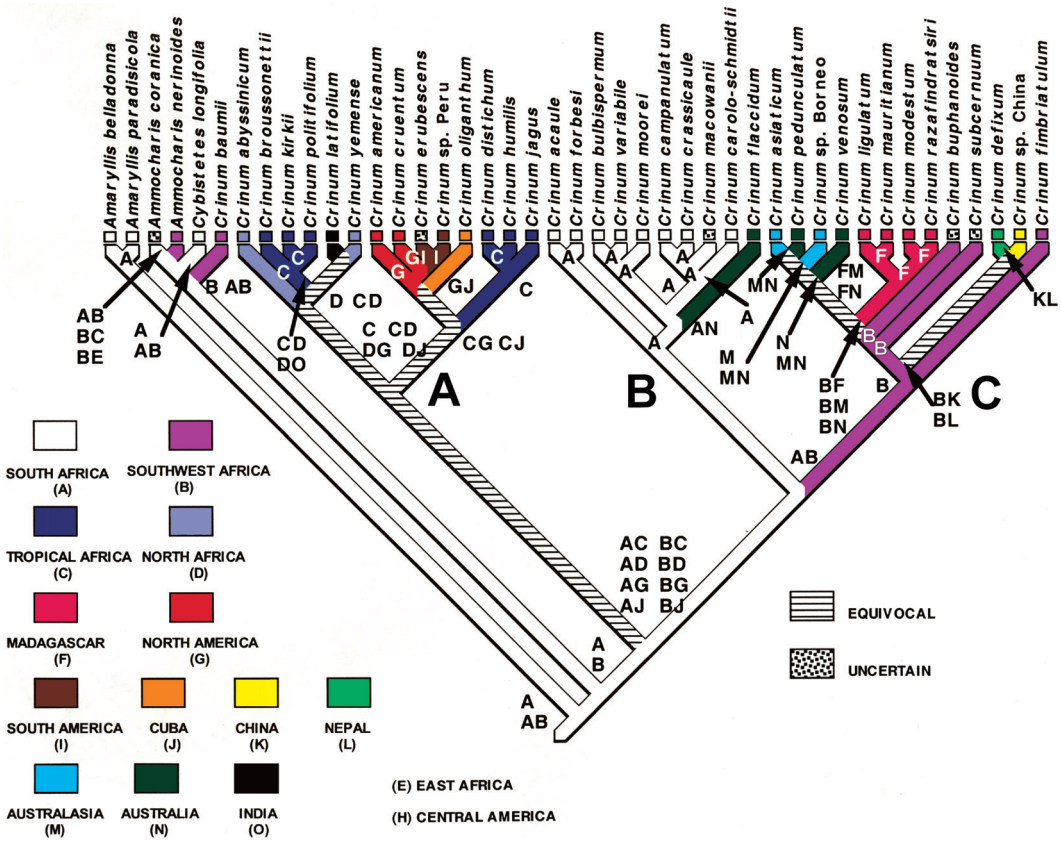

Figure 4. One of 150 equally most-parsimonious trees found by phylogenetic analysis of combined nrDNA ITS and plastid *trnL-F* sequences across 43 species of *Crinum* and related genera showing optimization of bigeographical data. Fitch optimization is indicated by colour or pattern; divergence-vicariance optimization is coded by small letters at ancestral nodes. Central America and East Africa are not visible in the Fitch optimization because they figure only in ambiguous area optimizations of terminal taxa.

easily modified. The sister group to *Crinum* (*Ammocharis* and *Cybistetes*; Meerow & Snijman, 2001) has zygomorphic perianths. However, *Crinum baumii*, which resolves strongly as part of the sister group to *Crinum*, has floral morphology typical of subg. *Crinum*. *Amaryllis*, sister to all remaining genera of tribe Amaryllideae (Meerow & Snijman, 2001), has zygomorphic perianths. Fitch optimization of this character onto a tree from our combined analysis (Fig. 5) suggests that actinomorphy is the apomorphic state and has evolved several times, once within the clade inclusive of the Asian and Madagascar species, once in the American clade, and yet again in the sister clade to *Crinum s.s.* Both *C. buphanoides* and *C. subcernuum* have subg. *Crinum*-type flowers, though their exact relationships to both the Malagasy and Asiatic clades are not resolved (Figs 1,3). There are only two species from tropical Africa with subg. *Crinum* floral morphology, *C. natans* Baker and *C. purpurascens* Herb., which unfortunately were not available for sequencing. They may well represent the African sister group to the American clade. Interestingly, both species are emergent aquatics as are most of the American species.

Fangan & Nordal (1993) also referred to a plastid RFLP-supported connection between East African (*C. macowanii* and *C. politifolium*) and 'Indo-Pacific' (*C. asiaticum*) *Crinum*. As no bootstrap or other confidence estimate was provided, the relative robustness of this clade could not be determined. However, our much larger sampling of species resolves *C. macowanii* and *C. politfolium* each in two clades, respectively (A and B, Figs 1,3,4) other than the one containing all of the Asian species that we sampled (clade C, Figs 1,3,4). The African mainland species within clade C wherein all of the Asian (both Sino-Himalayan and Australasian) species resolve are south-western and southern African in the main (*C. subcernuum*, described from Mozambique, has been collected in Namibia by the second author, and was collected once in Tanzania according to Nordal, 1977).

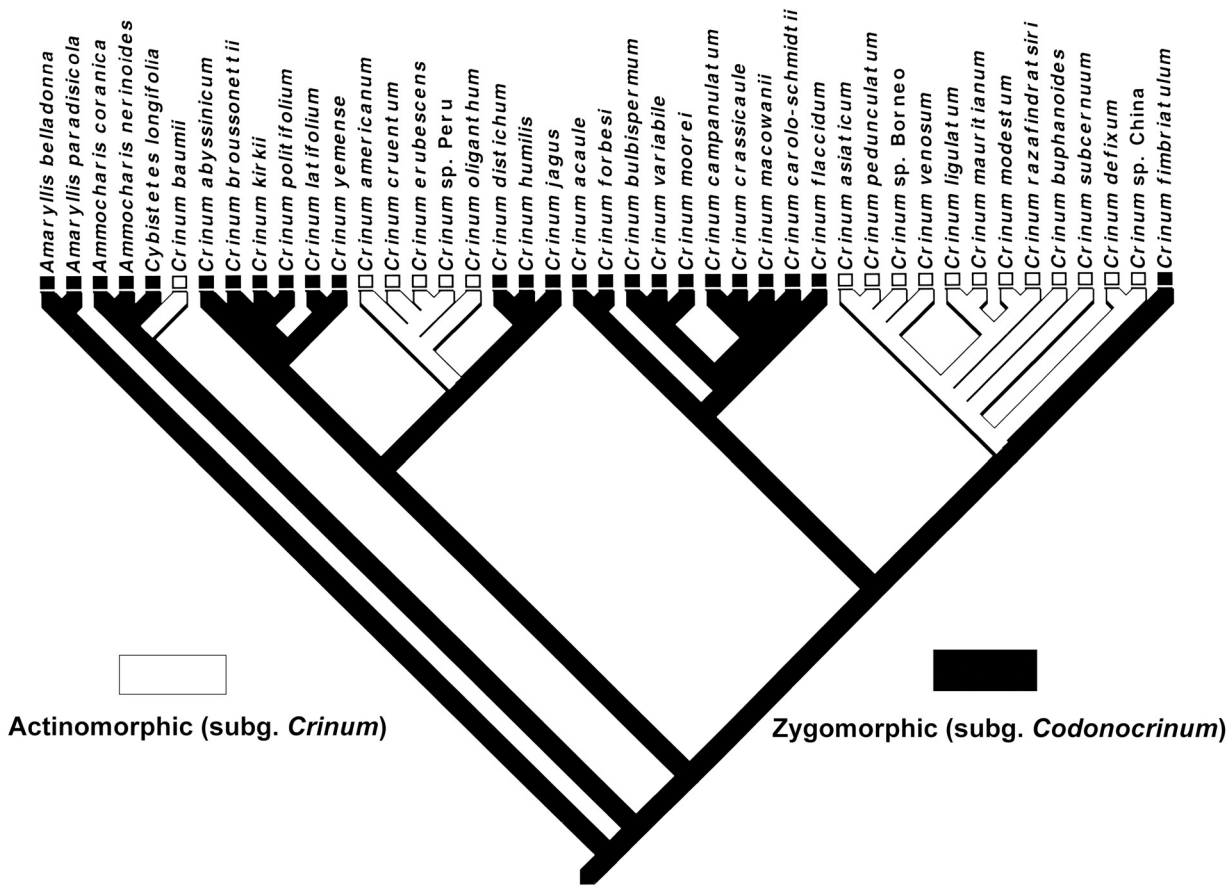

Figure 5. Fitch optimization of perianth morphology on one of 150 equally most-parsimonious trees found by phylogenetic analysis of combined nrDNA ITS and plastid *trnL-F* sequences across 43 species of *Crinum* and related genera.

Crinum baumii is sister to *Ammocharis* and *Cybistetes* with bootstrap support of 100% and DI = 11 (Figs 1,4). Milne-Redhead & Schweickerdt (1939) transferred *Crinum baumii* into *Ammocharis* without any detailed justification, and also established the monotypic genus *Cybistetes* as a segregate from the latter, largely on the basis of infructescence structure. In *Cybistetes*, the entire infructescence of indehiscent fruits functions as the dispersal unit (anemogeochory of Van der Pijl, 1982), whereas in *Ammocharis* the fruits are dehiscent and the infrutescence lax (Snijman & Williamson, 1994). Both *Ammocharis* and *Cybistetes* have biflabellate leaves, which *C. baumii* lacks, and zygomorphic perianths. Snijman & Williamson (1994) went so far as to suggest that separation of *Ammocharis* and *Cybistetes* needed re-examination. To this, we would add that the position of *C. baumii* relative to these two genera also requires re-evaluation, though there is little question that the species lies outside of *Crinum s.s.* based on the ITS phylogeny. This well-supported sister clade to *Crinum* is rooted within southern Africa (Fig. 4), but is broadly dispersed through sub-Saharan Africa via the five species of *Ammocharis*.

Given the likely role of oceanic dispersal of *Crinum* seed in the evolution of the genus, it is not surprising that several geographically cohesive clades are resolved in the genus by ITS and *trnL-F* sequences.

The roots of the entire lineage are in southern Africa, and area analyses places either South Africa (eastern South Africa in the case of *Crinum s.s.*) or south-western Africa within the ancestral node of all three clades of *Crinum* (Fig. 4). Each of the clades contains at least one dispersal event out of Africa (albeit only one in clade B), while clade B also encompasses continuous and perhaps recent evolution in South Africa. A monophyletic American clade denotes a single dispersal event into the Western Hemisphere. A monophyletic Madagascar group indicates a similar scenario. Two Asiatic clades are resolved as well, though not as sister groups, suggesting a possible double entry into the region.

A more precise understanding of the exact biogeographical scenario for Clade A is still elusive. The

absence of any southern African *Crinum* species in the American/North African/tropical African clade, if not an artifact of our sampling biases, may indicate that this clade represents the earliest vicariant divergence from the rest of the genus (Fig. 4). Ostensibly, the decimation of the tropical flora of Africa was a major isolating factor for the genus. Direct connections between South Africa and tropical Africa were most likely severely interrupted by the late Oligocene/early Miocene as uplift and climatic change began to create the modern landscape of the continent (Axelrod & Raven, 1978; Goldblatt, 1978; Coetzee, 1993). The apparent relationships of American species of *Crinum* with species from North and tropical Africa, rather than with the southern African elements of the genus (Fig. 4), might indicate that the dispersal point of departure from Africa to America was in the northern half of the African continent. A land bridge did exist between the Brazilian Bulge and Nigeria until the end of the Cretaceous (Rand & Mabesoone, 1982). Again, sister group relationships are obscured by a basal trichotomy in the American/North African/tropical African clade (Figs 1,3).

Clade C also presents some intriguing biogeographical hypotheses. One subclade represents an Australasian group, i.e. species found in south-east Asia, the Pacific and Australia (Fig. 4). A well-supported group within this subclade as presently sampled (*C. asiaticum*; *C. pedunculatum*, and *C*. sp., Borneo) is marked morphologically by the obsolescence of the bulb (the leaves basally form a tight and stout pseudostem which originates directly from a hyper-developed basal plate). The second, or Sino-Himalayan clade, represents species from the Himalayan region and nearby south-west China that form true bulbs, which may have entered the Indian subcontinent either directly from Africa or via Madagascar. Both dispersal pathways have been hypothesized for various taxa of angiosperms (Raven & Axelrod, 1974). The Sino-Himalayan clade barely receives moderate bootstrap support (65%) and has a DI of only 1. Its ancestral node has an equivocal Fitch optimization of area, and two possible area assignments from DIVA. Moreover, in the strict consensus tree of ITS alone it forms a polytomy with the Australasian clade, the Madagascar clade, *C. buphanoides* and *C. subcernuum* (both African and with subg. *Crinum* floral morphology); thus the sister group relationships of either Asian clade are not as clear as the single tree used for area optimization would indicate (Fig. 4). However, in the bootstrap consensus tree of the ITS matrix and in the combined analysis, the Sino-Himalayan clade is sister to an Australasian–Madagascar–*C. buphanoides*–*C. subcernuum* polytomy (Fig. 1, bootstrap = 50%; Fig. 3, bootstrap = 63%), suggesting an earlier dispersal event from Africa, with a likely initial entry into India. Consequently, dispersal to Australasia may have been an independent and later event.

Van Steenis (1962) hypothesized a land-bridge connection between Madagscar and Sri Lanka incorporating the Seychelles–Comores bank during the mid- to late Cretaceous, which he named 'Lemuria.' He saw no other way to account for plant distributions that encompassed the periphery of the Indian Ocean. Mckenzie & Sclater (1973) refuted the possibility. However, Haq, Hardenbol & Vail (1988) reported on the likeliness of increased emergence of the Chagos/Laccadive Plateau and then contiguous Mascarene Plateau (including the Seychelles Bank) during the early Oligocene (*c*. 30 MYA). Schatz (1996), in his review of the Indo-Australo-Malesian relationships of the Malagasy flora, postulates that lower sea levels allowed these emergences to function as stepping stones. Schatz's (1996) 'Lemurian stepping-stones' could have engendered migration of *Crinum* from Madgascar to western Malaysia. This may have also been the pathway by which the ancestor of *C. latifolium* dispersed from Africa to Sri Lanka and India. Emergent archipelagos may have existed, bridging much of the Indian Ocean between India and Australia as well (McKenzie & Sclater, 1973), which could have allowed dispersal of *Crinum* to northern Australia and from there into south-eastern Asia and the Pacific. *Crinum modestum* (Madagascar) and *C. venosum* (Australia) have a further morphological character in common; both have very short stamens that are atypical for subg. *Crinum*. Beyond coarse estimates, the relative timing of these events cannot be inferred, again due to the internal trichotomy formed by the major subclades of clade C (Figs 1,3).

It is also evident that *Crinum* entered the Australian continent at least twice. The other migration (*C. flaccidum*, '*Codonocrinum*') was apparently directly from Africa (in clade B, Fig. 4). The African relationships of the more southern Australian *Crinum flaccidum* are also resolved by both ITS and *trnL-F* (Figs 1,2). If homoplasious base substitutions in the ITS alignment are down-weighted using successive approximation (Farris, 1969; Wenzel, 1997; Lledó *et al*., 1998; Meerow *et al*., 1999), a sister relationship between these two species is also resolved (data not shown). Both species share similar seasonally aquatic habitats (albeit on different continents) and have terete juvenile leaves. Moreover, like *C. flaccidum*, the eastern Cape endemic *C. campanulatum*, has a campanulate perianth, but not declinate stamens. In the combined analysis (Fig. 3), the position of *C. flaccidum* as sister to an otherwise African clade is better supported than by ITS alone.

How did *Crinum* get to Australia this first time, assuming an early introduction for the ancestor(s) of *C. flaccidum*, all of whose closest extant relatives are

endemic to Africa? Three pathways are possible. Oceanic long distance dispersal directly from Africa is one scenario. Secondly, Madagascar–India could have provided an intermediate route, followed by oceanic dispersal to Australia, *c*. 65 MYBP (Raven & Axelrod, 1974). However, the phylogenetic relationships of *C. flaccidum* are not with any extant Madagascar or Indian *Crinum* species (Fig. 4). It is certainly possible that the extant Madagascar and Indian species represent more recent migrations and that the earlier migratory ancestors of *C. flaccidum* are extinct. A third possible migration could have been via a more southerly subtropical route through Antarctica, that by traditional geophysical hypotheses (Smith & Hallam, 1970; Smith, Smith & Funnell, 1994) was available too early in the diversification of the angiosperms to have figured in the biogeographical history of a higher asparagoid monocot. However, more recent hypotheses based on palaeontological evidence (Sampson *et al*., 1998; Hay *et al*., 1999; Krause *et al*., 1999) suggest that a longer-lived land connection between India and Antarctica–Australia via the Kerguelen Plateau may have existed as late as 80 MYBP.

Outside of two distinct clades of *Crinum* in Australia, the continent is home to a distinctive tribal clade of the family (Meerow & Snijman, 1998; Meerow *et al*., 1999), the Calostemmateae (*Proiphys* Herb. and *Calostemma* R. Brown, the latter endemic to Australia, the former also extending into south-east Asia). In the case of Calostemmateae, one might infer that the tribe originated in Australia and later migrated to south-east Asia, since the continent contains both the generic and species diversity of the lineage. The sister relationships of this tribe have still not been resolved (Meerow *et al*., 1999). In the lower asparagoid family Iridaceae, all species of the genus *Dietes* are endemic to eastern through southern sub-Saharan Africa with the exception of a single Australian species on Lord Howe Island (Goldblatt, 1981) that is considered the least morphologically derived species in the genus (Goldblatt, 1978). Baum, Small & Wendel (1998) concluded that dispersal between Africa and Australia in the genus *Adansonia* (Malvaceae) occurred via ocean currents considerably after the break-up of Gondwanaland. In the case of *Adansonia*, the sole Australian species was sister to the rest of the genus (Madagascar and Africa), thus the direction of the dispersal event was ambiguous. In *Crinum*, there is no ambiguity that the genus originated in Africa (Fig. 4).

Southern Africa is also where the sister group to *Crinum* originated (Fig. 4), and is the centre of diversity for the genus (Nordal, 1977; Fangan & Nordal, 1993). This is not to suggest that all of this diversity is necessarily ancient; it may reflect radiation engendered by the more recent palaeoclimatic and geological history of Africa encompassing Neogene and later times (Axelrod, 1972; Raven & Axelrod, 1974; Coetzee, 1993). The increased aridity of the African climate and the uplift of the continental mass that started at the beginning of the Miocene, further abetted by Quaternary climatic fluctuations (Demenocal, 1995) were catastrophic to many elements of the African flora, but it may have been a selective pressure for diversity among groups of geophytes capable of adapting to increasing drought. The geophyte richness of South Africa is well documented (Goldblatt, 1978), and the Cape region has been suggested as a possible refuge for certain African plant and animal groups as the tropical flora of the continent was impoverished (Raven & Axelrod, 1974). *C. variabile* is the only *Crinum* species so far known from the winter-rainfall region of western South Africa (Verdoorn, 1973). Subtropical forests were still present in the Western Cape during the Miocene and Pliocene (Scott *et al*., 1997). The earliest evidence of modern semiarid, winter-rainfall pattern in the Western Cape dates to the Late Pliocene, but it was not fully established until the Early Pleistocene (Tankard & Rogers, 1978; Hendey, 1983; Coetzee, 1986). Moreover, the winter-rainfall region of southern Africa experienced a more recent pattern of expansion and contraction with concurrent wetter and drier conditions during glacial and interglacial periods of the Quaternary (Tankard, 1976; van Zinderen Bakker, 1976; Tyson, 1986; Crockcroft, Wilkinson & Tyson, 1987). It would appear that a winter rainfall regime is largely inimical to *Crinum*. Nonetheless, it is impossible to determine if the genus ever existed in the Western Cape prior to the establishment of the Mediterranean climate.

In conclusion, nuclear rDNA ITS sequences support a southern African (eastern South Africa or southwestern Africa) origin for the genus *Crinum*, and indicate three major waves of radiation corresponding to the three main clades resolved in our trees (Figs 1,3,4). Two entries into Australia for the genus are hypothesized. Asian and Malagasy *Crinum* are phylogenetically related, and separate Sino-Himalayan and Australasian dispersals are indicated. The monophyletic American species are allied with tropical and North African species. Recognition of two subgenera in *Crinum* on the basis of floral morphology is not supported by the molecular phylogeny, as the apomorphic subg. *Crinum* floral morphology has evolved more than once (Fig. 5). *Crinum baumii* appears to be more closely related to *Ammocharis* and *Cybistetes*, and the taxonomic standing of this species and both of these genera needs to be re-evaluated in light of this relationship.

We sought to augment the incomple resolution of phylogenetic relationships within *Crinum* with data from the plastid *atpB-rbcL* intergenic spacer (Chiang,

Schaal & Peng, 1998). The AT-rich spacer proved extremely hyper-variable, and necessitated the trimming of 360 bp that was impossible to align, even within clades well-supported by ITS. The resulting matrix was poorly resolved (data not shown), but after successive weighting, a few clades in common with the ITS or *trnL-F* phylogeny were supported: (1) a tropical and North African clade (bootstrap = 88%), (2) a Madagascar/Asian clade (71%), albeit with the anomalous inclusion of *C. crassicaule*, (3) a *C. campanulatum*/*C. flaccidum* sister relationship (87%), and (4) a *C. kirkii–C. politifolium* clade (99%). We are in the process of obtaining sequences for the plastid *ndhF* gene, in the hopes that it will resolve the basal polytomies within the ITS phylogeny and allow stratigraphic estimation of divergence within the genus.

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